Acid Sphingomyelinase Regulates Platelet Cell Membrane Scrambling, Secretion, and Thrombus Formation

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Objective—Platelet activation is essential for primary hemostasis and acute thrombotic vascular occlusions. On activation, platelets release their prothrombotic granules and expose phosphatidylserine, thus fostering thrombin generation and thrombus formation. In other cell types, both degranulation and phosphatidylserine exposure are modified by sphingomyelinase-dependent formation of ceramide. The present study thus explored whether acid sphingomyelinase participates in the regulation of platelet secretion, phosphatidylserine exposure, and thrombus formation.

Approach and Results—Collagen-related peptide–induced or thrombin-induced ATP release and P-selectin exposure were significantly blunted in platelets from Asm-deficient mice (*Smpd1*$^{-/-}$) when compared with platelets from wild-type mice (*Smpd1*$^{+/+}$). Moreover, phosphatidylserine exposure and thrombin generation were significantly less pronounced in *Smpd1*$^{-/-}$ platelets than in *Smpd1*$^{+/+}$ platelets. In contrast, platelet integrin $\alpha_{IIb}\beta_3$ activation and aggregation, as well as activation-dependent Ca^{2+} flux, were not significantly different between *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ platelets. In vitro thrombus formation at shear rates of 1700 s^{-1} and in vivo thrombus formation after $FeCl_3$ injury were significantly blunted in *Smpd1*$^{-/-}$ mice while bleeding time was unaffected. Asm-deficient platelets showed significantly reduced activation-dependent ceramide formation, whereas exogenous ceramide rescued diminished platelet secretion and thrombus formation caused by Asm deficiency. Treatment of *Smpd1*$^{+/+}$ platelets with bacterial sphingomyelinase (0.01 U/mL) increased, whereas treatment with functional acid sphingomyelinase-inhibitors, amitriptyline or fluoxetine (5 μmol/L), blunted activation-dependent platelet degranulation, phosphatidylserine exposure, and thrombus formation. Impaired degranulation and thrombus formation of *Smpd1*$^{-/-}$ platelets were again overcome by exogenous bacterial sphingomyelinase.

Conclusions—Acid sphingomyelinase is a completely novel element in the regulation of platelet plasma membrane properties, secretion, and thrombus formation. **(*Arterioscler Thromb Vasc Biol*. 2014;34:61-71.)**

Key Words: acid sphingomyelinase ■ amitriptyline ■ granule secretion ■ phosphatidylserine exposure ■ platelets ■ thrombus formation ■ thrombin generation

Platelets are critically important for primary hemostasis after vascular injury and are pivotal elements in the development of acute thrombotic occlusion and subsequent myocardial infarction and ischemic stroke.[1,2] Moreover, platelets may contribute to the pathophysiology of disorders not directly related to hemostasis or thrombosis, such as cancer,[3] inflammation,[4,5] host–pathogen interaction,[5,6] and lymphatic development.[7] Platelets are activated by a wide variety of stimulators, such as subendothelial collagen, ADP released from activated platelets, or activated thrombin and collagen-related peptide (CRP).[8–10] After activation, platelets secrete their granules, undergo cell membrane scrambling with phosphatidylserine exposure, aggregate, and form thrombi resulting in vascular occlusion.[11] Thereby phosphatidylserine exposure at the outer membrane surface provides binding sites for coagulation factors and promotes formation of factor Xa and thrombin by facilitating assembly of tenase and prothrombinase complexes.[12,13]

In other cell types, cell membrane scrambling[14,15] and degranulation[16] are modified by ceramide producing acid sphingomyelinase (ASM). Sphingomyelinases break down membrane sphingomyelin with ceramide production and play

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Nonstandard Abbreviations and Acronyms	
ASM	acid sphingomyelinase
bSM	bacterial sphingomyelinase
CRP	collagen-related peptide

an important role in lipid signaling of different cardiovascular diseases resulting in vascular inflammation.[17] Ceramides form ceramide-rich platforms thus increasing membrane curvature and eventual fusion and fission of vesicles.[18] Ceramide participates in vascular inflammation and thrombosis by triggering exocytosis of Weibel–Palade bodies from endothelial cells, an effect inhibited by exogenous NO.[19] ASM is a key regulator of T-lymphocyte granule secretion, degranulation, vesicle shedding, and especially phosphatidylserine-exposing microparticle release from glial cells.[16,20] By producing ceramide, ASM may trigger cell membrane scrambling with phosphatidylserine exposure and thus suicidal death of other blood cells, such as erythrocytes.[21,22]

ASM activity and ceramide formation thus contribute to the pathophysiology of a wide variety of disorders, including atherosclerosis,[23–25] inflammation, fibrosis and infection,[26] cystic fibrosis,[26–28] Wilson disease,[22] diabetes mellitus,[29] cardiovascular disease,[30,31] cerebral ischemia,[32] multiple sclerosis[33] major depression,[14] Parkinson disease,[34] and Alzheimer disease.[14] Tricyclic antidepressant medications, such as amitriptyline or fluoxetine, are widely used as experimental functional ASM-inhibitors.[35]

Although it is known that platelets secrete ASM on thrombin stimulation,[36,37] nothing is known about the role of ASM in platelet physiology. The present study thus explored the role of ASM in platelet function. To this end, platelets have been isolated from gene-targeted mice lacking functional ASM (*Smpd1*$^{-/-}$) and from their wild-type littermates (*Smpd1*$^{+/+}$).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

To study the functional role of ASM in the regulation of platelet function, platelets were isolated from gene-targeted mice lacking functional Asm (*Smpd1*$^{-/-}$) and their respective wild-type littermates (*Smpd1*$^{+/+}$).

Smpd1$^{-/-}$ mice did not exhibit spontaneous bleeding. Blood platelet counts and mean platelet volume were similar in *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ mice (Table), indicating that Asm is not essential for platelet generation. No differences were found in other hematologic parameters (Table). Furthermore, transmission electron microscopy of Asm-deficient platelets revealed no significant morphological differences and especially no difference in number and morphology of α-granules and dense granules when compared with platelets from wild-type mice (Figure I in the online-only Data Supplement).

To elucidate the effect of Asm on platelet secretion, the activation-dependent release of platelet α (P-selectin exposure) and dense granules (ATP release) was quantified before and after platelet stimulation with CRP or thrombin. As illustrated in Figure 1A, P-selectin abundance at the platelet surface after stimulation with low concentrations of CRP (0.1 and 1.0 μg/mL) and thrombin (0.005 U/mL) was significantly lower in *Smpd1*$^{-/-}$ platelets than in *Smpd1*$^{+/+}$ platelets. Degranulation after stimulation with high concentrations of CRP (5 μg/mL; data not shown) or thrombin (0.02 U/mL) was not significantly different. To explore the effect of Asm on degranulation of platelet dense granules further, activation-dependent ATP release was determined in *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets. The increase of ATP concentration in the supernatant after stimulation with lower concentrations of CRP (0.5 and 1 μg/mL) or thrombin (0.005 and 0.02 U/mL) was significantly diminished in *Smpd1*$^{-/-}$ platelets as compared to *Smpd1*$^{+/+}$ platelets (Figure 1C and 1D). At high CRP (≥5 μg/mL) or thrombin (0.1 U/mL) concentrations, no significant difference was found.

Table. Blood Count of *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ mice

	Smpd1$^{+/+}$	*Smpd1*$^{-/-}$
Platelets, $\times 10^3/\mu L$	1097±39	1105±108
MPV, fl	6.6±0.1	6.4±0.1
Erythrocytes, $\times 10^6/\mu L$	7.7±0.7	7.9±0.5
HGB, g/dL	11.2±1.1	11.8±0.9
HCT, %	37.2±4.6	38.2±4.0
MCV, fl	48.4±1.8	49.2±1.5

Arithmetic mean±SEM (n=8) of platelet count, mean platelet volume (MPV), red blood cell (RBC) count (erythrocytes), hemoglobin concentration (HGB), hematocrit (HCT), and mean RBC volume (MCV).

Although the activation-dependent platelet secretion was significantly diminished in Asm-deficient platelets, integrin $\alpha_{IIb}\beta_3$ activation (Figure 1B) and aggregation (Figure 1E and 1F), after low dose and high dose of thrombin or CRP stimulation were not significantly different in *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets.

To examine the role of Asm in activation-dependent platelet phosphatidylserine exposure after cell membrane scrambling and induction of the coagulation cascade by activation of prothrombinase complexes, phosphatidylserine exposure (annexin-V binding) and thrombin generation were quantified before and after platelet stimulation (Figure 2). The effect of Asm on activation-dependent platelet cell membrane scrambling with procoagulant phosphatidylserine exposure was determined by flow cytometric analysis of platelet annexin-V binding after stimulation with thrombin (1 U/mL) or a combination of thrombin (0.01 U/mL) and CRP (5 μg/mL). As shown in Figure 2A, activation-dependent phosphatidylserine exposure was significantly blunted in Asm-deficient platelets than in wild-type platelets. In a further series of experiments, we found that thrombin generation is significantly affected in *Smpd1*$^{-/-}$ platelets. As illustrated in Figure 2B through 2D, platelet-rich plasma from Asm-deficient mice displayed significantly reduced peak levels of thrombin generation, whereas thrombin levels in platelet-poor plasma were similar in *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ mice.

Platelet activation, degranulation, and subsequent thrombus formation are triggered by an increase of intracellular

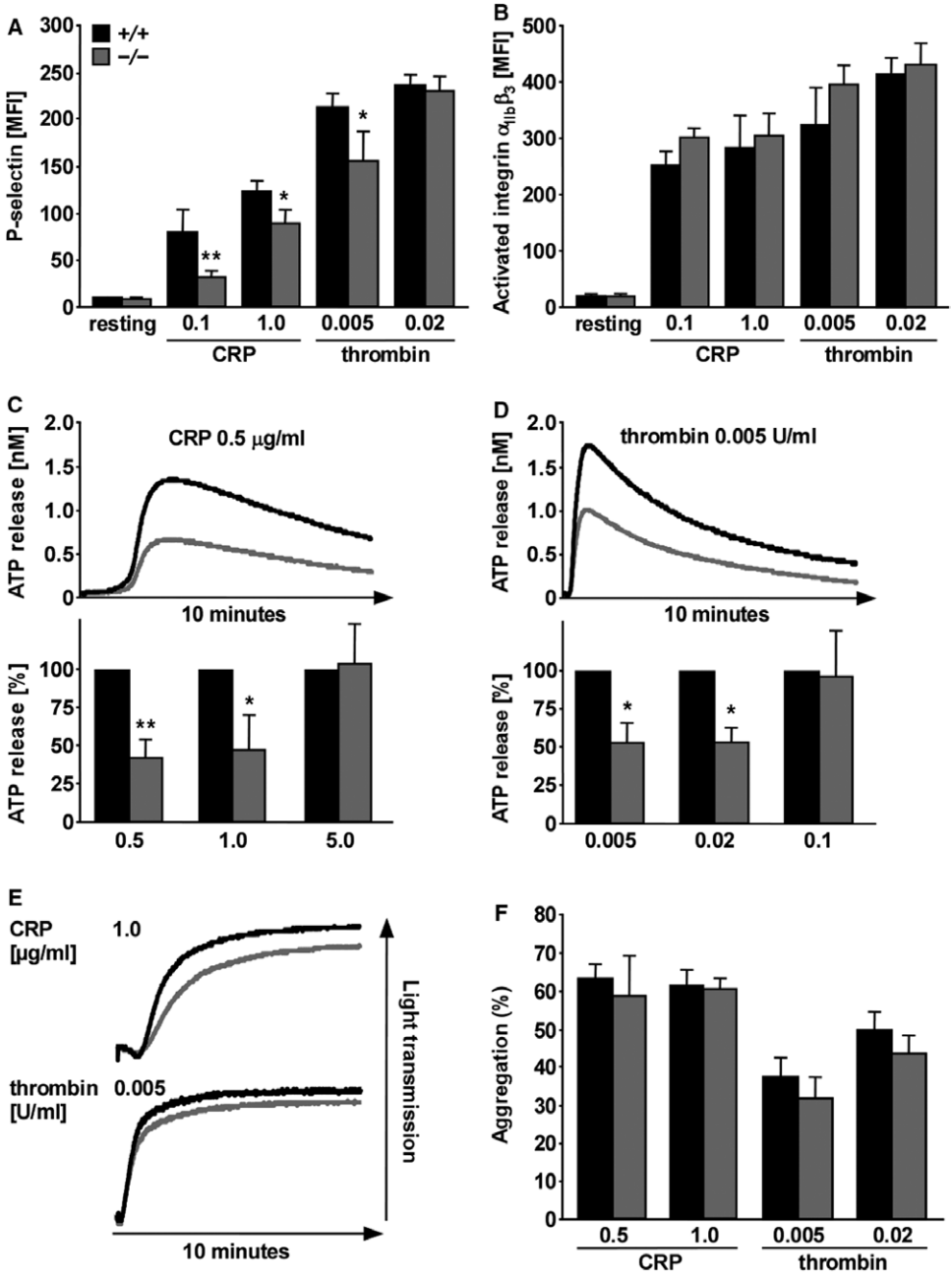

Figure 1. Activation-dependent release of α-granules and dense granules, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation in platelets from *Smpd1*$^{-/-}$ and *Smpd*$^{+/+}$ mice. **A**, Arithmetic means±SEM (n=6) of P-selectin exposure determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in response to collagen-related peptide (CRP; μg/mL) or thrombin (U/mL) at the indicated concentrations. **B**, Arithmetic means±SEM (n=6) of activated integrin $\alpha_{IIb}\beta_3$ determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in response to CRP (μg/mL) or thrombin (U/mL) at the indicated concentrations. **C**, CRP-dependent ATP release from *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets. Original tracings (**top**) and arithmetic means±SEM (n=6; **bottom**) illustrating ATP concentration in the supernatant after stimulation of platelets from either *Smpd1*$^{+/+}$ (black bars) or *Smpd1*$^{-/-}$ (gray bars) mice with CRP. **D**, Thrombin-dependent ATP release from *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets. Original tracings (**top**) and arithmetic means±SEM (n=6; **bottom**) illustrating the ATP concentration in the supernatant after stimulation of platelets from either *Smpd1*$^{+/+}$ (black) or *Smpd1*$^{-/-}$ (gray) mice with thrombin. **E**, Aggregation: representative tracings of aggregometry after stimulation of platelets from either *Smpd1*$^{+/+}$ (black) or *Smpd1*$^{-/-}$ (gray) mice with either 1 μg/mL CRP (**top**) or 0.005 U/mL thrombin (**bottom**). **F**, Arithmetic means±SEM (n=6) of aggregometry after stimulation of platelets from either *Smpd1*$^{+/+}$ (black bars) or *Smpd1*$^{-/-}$ (gray bars) mice with either CRP (μg/mL) or thrombin (U/mL) at the indicated concentrations. **P*<0.05 and ***P*<0.01 indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets.

Ca^{2+} concentration ($[Ca]^{2+}_i$), which is accomplished by Ca^{2+} store depletion and store operated Ca^{2+} entry. In theory, ASM could have an effect on degranulation, phosphatidylserine exposure, and thrombus formation by modifying $[Ca]^{2+}_i$. However, spectrofluorimetric measurements revealed that activation-dependent $[Ca]^{2+}_i$ increases were similar in *Smpd1*$^{-/-}$ platelets and in *Smpd1*$^{+/+}$ platelets after stimulation with CRP or thrombin (Figure IIB in the online-only Data

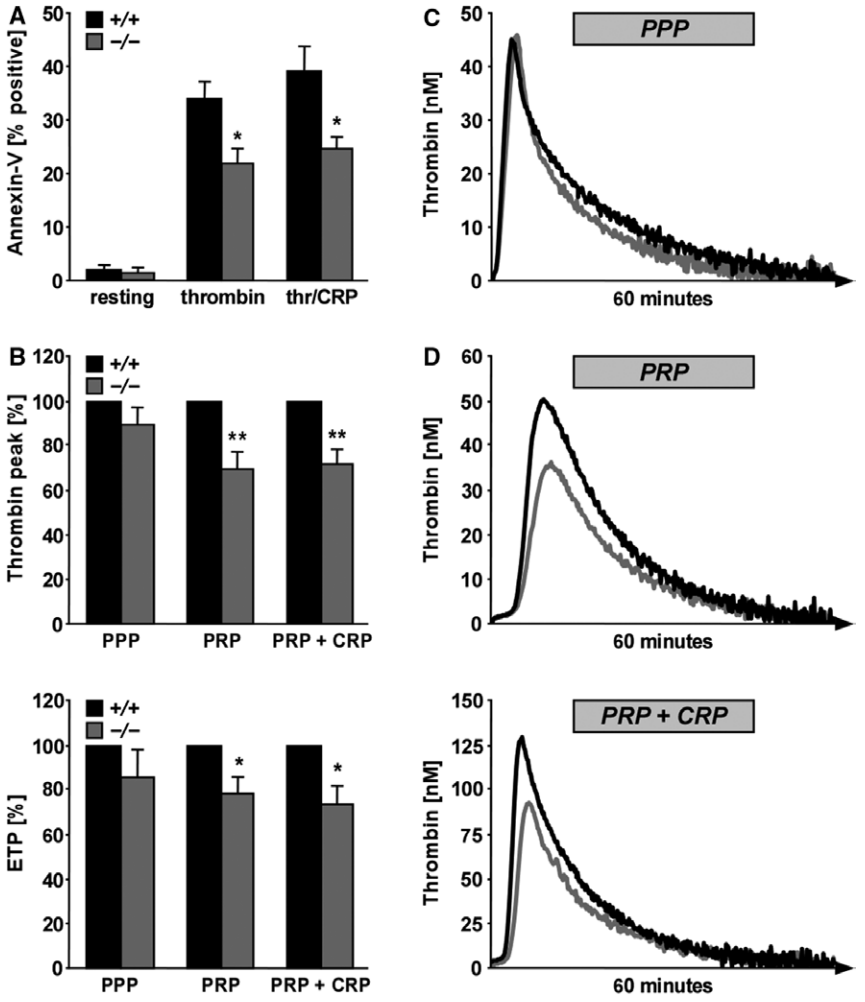

Figure 2. Activation-dependent procoagulant activity of *Smpd1*$^{-/-}$ and *Smpd*$^{+/+}$ platelets. **A**, Activation-dependent phosphatidylserine exposure of *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets. Arithmetic means±SEM (n=6) of phosphatidylserine exposure (annexin-V) determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in response to thrombin (thr) (1.0 U/mL) and thrombin/collagen-related peptide (CRP; 0.01 U/mL/5 μg/mL). **B**, Activation-dependent thr generation of *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) platelets. Arithmetic means±SEM (n=6) of thr peak and endogenous thrombin potential (ETP) (% from wild type) in platelet-poor plasma (PPP) and platelet-rich plasma (PRP) in the presence or in the absence of CRP (5 μg/mL). **C**, Representative thr generation tracings in PPP from either *Smpd1*$^{+/+}$ (black) or *Smpd1*$^{-/-}$ (gray) mice. **D**, Representative thr generation tracings in PRP from either *Smpd1*$^{+/+}$ (black) or *Smpd1*$^{-/-}$ (gray) mice in the presence (**bottom**) or in the absence (**top**) of CRP (5 μg/mL). **P*<0.05 and ***P*<0.01 indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets.

Supplement). Furthermore, as shown in Figure IIA in the online-only Data Supplement, Asm deficiency did not appreciably influence intracellular Ca^{2+} release or store operated Ca^{2+} entry induced in *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ platelets with sarco/endoplasmic reticulum (SR/ER) Ca^{2+} ATPase pump inhibitor thapsigargin.

To elucidate the relevance of Asm in pathological thrombus formation, we examined in vitro platelet adhesion and thrombus formation to collagen-coated surfaces under flow at low (500 s^{-1}) and high (1700 s^{-1}) wall shear rates. As illustrated in Figure 3A, *Smpd1*$^{+/+}$ platelets formed massive and dense thrombi after 5 minutes of perfusion. At shear rates of 500 s^{-1}, no significant reduction of thrombus formation was found, but at high arterial shear rates *Smpd1*$^{-/-}$ platelets formed only smaller single thrombi with a significantly reduced thrombus surface coverage (Figure 3A and 3B). Real-time imaging of thrombus formation under flow by capturing images of fluorescent-labeled platelets indicated a consistent and gradual increase in fluorescence intensity from deposited *Smpd1*$^{+/+}$ platelets (Figure 3E). With *Smpd1*$^{-/-}$ blood, the slope of fluorescence increase was diminished, and time traces pointed to a slow, but gradual thrombus buildup. Notably, time traces from none of the experiments or inspection of consecutive images showed evidence for increased thrombus instability or embolization events with *Smpd1*$^{-/-}$ blood.

To assess the significance of Asm for arterial thrombus formation in vivo, time to occlusion of mesenteric arterioles caused by thrombus formation after $FeCl_3$-induced injury have been analyzed. As shown in Figure 3C and 3D, vascular occlusion caused by arterial thrombus formation was significantly impaired in *Smpd1*$^{-/-}$ mice. Nevertheless, the tail bleeding time was nearly unaffected in Asm-deficient mice (Figure 3F).

Because Asm has been identified as critical enzyme regulating ceramide production, we examined the activation-dependent ceramide formation in *Smpd1*$^{-/-}$ platelets. As a result, we found significantly impaired ceramide formation in Asm-deficient platelets when compared with wild-type platelets on stimulation with thrombin or the combination of thrombin and CRP (Figure 4A). In a further series of experiments, we tried to restore the ceramide defect in *Smpd1*$^{-/-}$ platelets by the addition of exogenous ceramide C16. As illustrated in Figure 4, addition of exogenous ceramide rescued the defective granule secretion (Figure 4B and 4C), as well as the blunted thrombus formation of *Smpd1*$^{-/-}$ platelets (Figure 4D and 4E) up to a level comparable with that of wild-type platelets.

In the next step, we explored the effect of pharmacological ASM inhibition with amitriptyline (5 μmol/L) in wild-type mouse platelets (Figure 5). The increase of α-granule

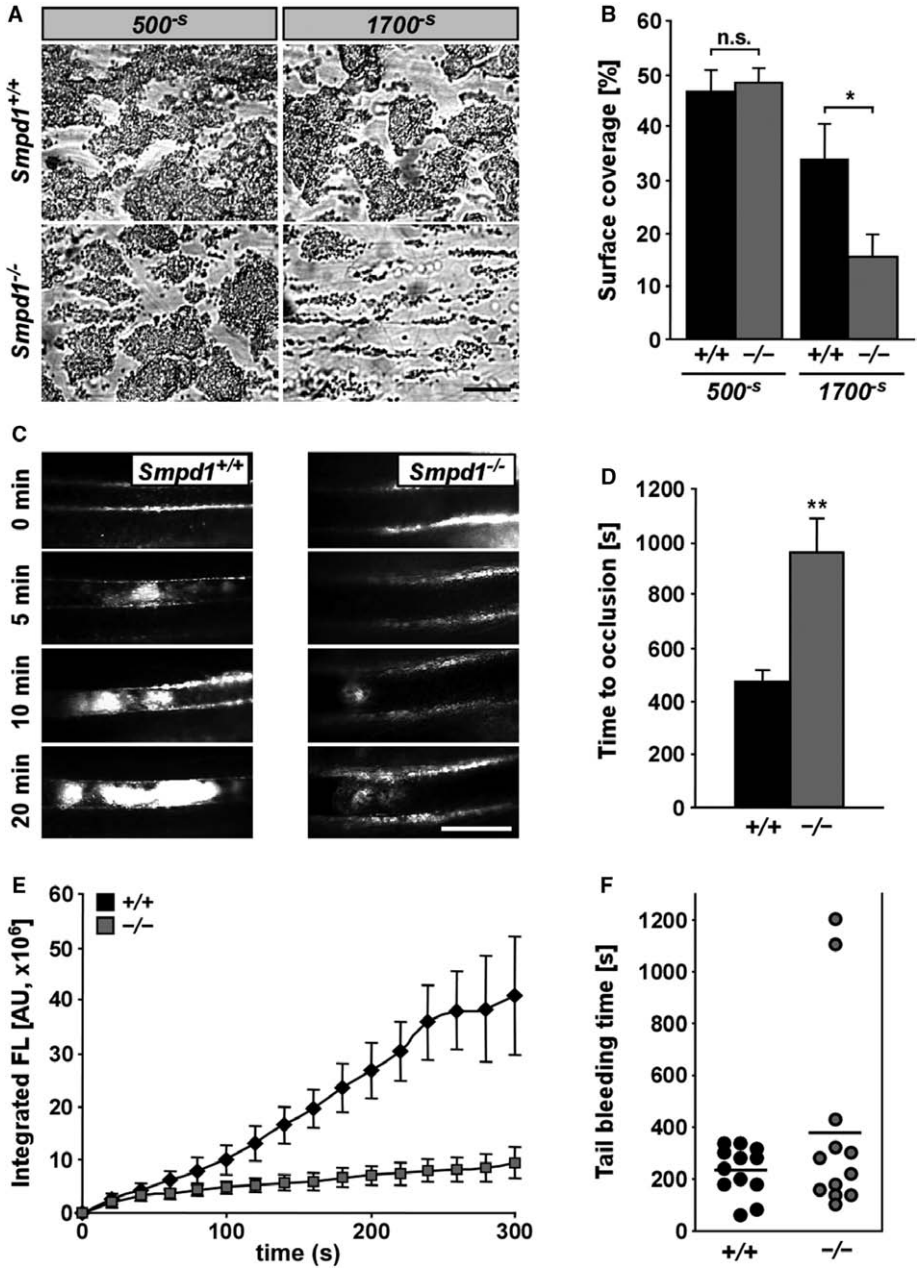

Figure 3. Formation and stability of thrombi formed by *Smpd1*$^{-/-}$ and *Smpd*$^{+/+}$ platelets, arterial thrombotic occlusion in vivo, and tail bleeding time in Smpd1$^{-/-}$ and Smpd$^{+/+}$ mice. **A**, Original phase-contrast images of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (**top**) and *Smpd1*$^{-/-}$ (**bottom**) mice over a collagen-coated surface for 5 minutes at low (500 s^{-1}, **left**) and high (1700 s^{-1}, **right**) arterial shear rates. Scale bar, 50 μm. **B**, Arithmetic means±SEM (n=5) of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice over a collagen-coated surface for 5 minutes at low (500 s^{-1}, **left**) and high (1700 s^{-1}, **right**) arterial shear rates. *P<0.05 indicates statistically significant difference. **C**, Representative images of occlusive in vivo thrombus formation 0, 5, 10, and 20 minutes after injury of mesenteric arterioles with $FeCl_3$ in *Smpd1*$^{+/+}$ (**left**) and *Smpd1*$^{-/-}$ (**right**) mice. Scale bar, 50 μm. **D**, Arithmetic means±SEM (n=10) of time to arterial occlusion after $FeCl_3$-induced injury of mesenteric arterioles in *Smpd1*$^{+/+}$ (black bar) and *Smpd1*$^{-/-}$ (gray bar) mice. **P<0.01 indicates statistically significant difference. **E**, Arithmetic means±SEM (n=4) of integrated fluorescence (FL) intensity of adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (black diamonds) and *Smpd1*$^{-/-}$ (gray diamonds) mice over a collagen-coated surface representing thrombus growth and stability in vitro >5 minutes. **F**, Tail bleeding time measured after amputating the tail tip of *Smpd1*$^{+/+}$ (black dots) and *Smpd1*$^{-/-}$ (gray dots) mice. Each dot represents 1 individual; black bar represents the mean value (n=12; P=0.21). n.s. indicates nonsignificant.

secretion (P-selectin exposure; Figure 5A) and dense granule secretion (ATP release; Figure 5C and 5D) after stimulation with lower concentrations of CRP (0.1 and 1 μg/mL) or thrombin (0.005–0.02 U/mL) was significantly decreased in the presence of the functional Asm inhibitor amitriptyline (5 μmol/L). At high CRP (≥ 5 μg/mL) or thrombin (0.1 U/mL) concentrations, degranulation tended to be lower in the presence than in the absence of amitriptyline; however, the difference did not reach statistical significance (data not shown). Phosphatidylserine exposure after platelet stimulation with thrombin (1.0 U/mL) or the combination of thrombin (0.01 U/mL) and CRP (5 μg/mL) was significantly diminished after preincubation with amitriptyline (5 μmol/L) when compared with solvent control (Figure 5B). Similar observations were made in arterial thrombus formation at shear rates of 1700 s^{-1} after inhibition of ASM with 5 μmol/L amitriptyline. As illustrated in Figure 5E, thrombi formed by *Smpd1*$^{+/+}$ platelets after 5-minute perfusion were significantly blunted in the presence of amitriptyline (5 μmol/L), whereas amitriptyline showed no further effect on reduced thrombus formation of *Smpd1*$^{-/-}$ platelets.

Nearly the same results as with amitriptyline were obtained with fluoxetine, a second functional pharmacological inhibitor of ASM (Figure 6). At concentrations of 5 μmol/L, fluoxetine inhibited activation-dependent platelet secretion at low agonist concentrations of thrombin (0.005 U/mL) and CRP (0.5 and 1.0 μg/mL), as well as the thrombus formation at high arterial shear rates (Figure 6C).

Opposite effects were observed in the presence of 0.01 U/mL bacterial ASM (Figure 7). The increase of P-selectin exposure (Figure 7A) and ATP release (Figure 7C and 7D) after stimulation with lower concentrations of CRP (0.1

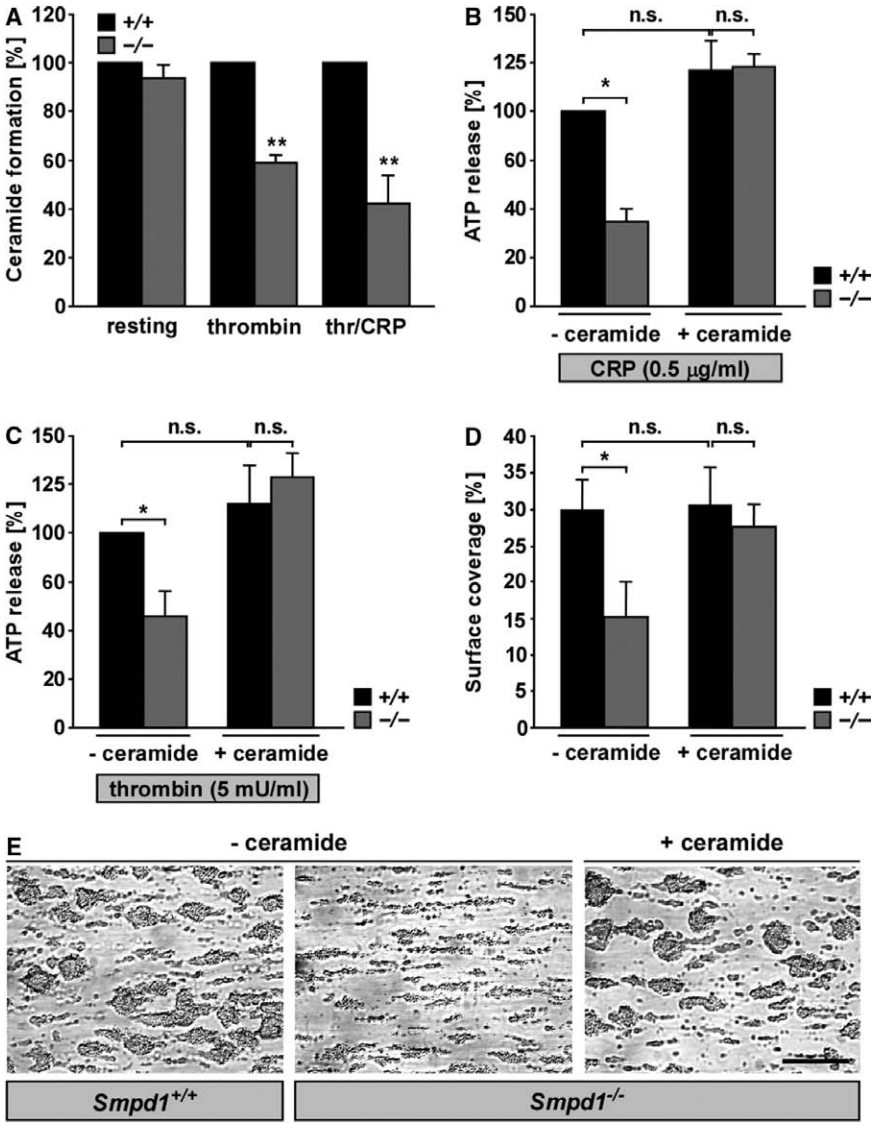

Figure 4. Ceramide formation and the role of ceramide in defective secretion and thrombus formation in *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ platelets. **A**, Activation-dependent ceramide production of *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) platelets after stimulation with thrombin (thr; 1 U/mL) or the combination of thr (0.1 U/mL) and collagen-related peptide (CRP; 5 μg/mL). Arithmetic means±SEM (n=6) of ceramide formation (% from wild type) in washed platelets are shown. **B**, Arithmetic mean±SEM (n=6) illustrating the ATP concentration in the supernatant after stimulation with 0.5 μg/mL CRP of platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or presence (**right**) of ceramide C16 (1 μmol/L). **C**, Arithmetic means±SEM (n=6) illustrating the ATP concentration in the supernatant after stimulation with 0.005 U/mL thr of platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or presence (**right**) of ceramide C16 (1 μmol/L). **D**, Arithmetic means±SEM (n=5) of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or presence (**right**) of 1 μmol/L ceramide C16 over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. **E**, Original representative phase-contrast images of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ mice in the absence or in the presence of 1 μmol/L ceramide C16 over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. Scale bar, 50 μm. ***P*<0.01 and **P*<0.05 indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets. n.s. indicates nonsignificant.

and 1 μg/mL) or thrombin (0.005 U/mL) were significantly increased in the presence of bacterial sphingomyelinase (bSM; 0.01 U/mL). At high CRP (≥ 5 μg/mL) or thrombin (0.1 U/mL) concentrations, degranulation again tended to be higher in the presence than in the absence of bacterial ASM; however, the difference did not reach statistical significance (data not shown). A significantly increased phosphatidylserine exposure was observed in thrombin-stimulated platelets (1.0 U/mL) or the combination of thrombin (0.01 U/mL)- and CRP (5 μg/mL)-stimulated platelets after treatment with bSM when compared with solvent control treatment (Figure 7B).

Exposure of the platelets to bSM (0.01 U/mL) significantly augmented platelet secretion, phosphatidylserine exposure, and formation of thrombi in *Smpd1*$^{-/-}$ platelets up to the values observed in wild-type platelets (Figure 8). In detail, bSM rescued defective activation-dependent ATP release on stimulation with CRP (0.5 μg/mL) or thrombin (0.005 U/mL) (Figure 8A and 8B), as well as CRP-/thrombin-triggered phosphatidylserine exposure (Figure 8C) and thrombus formation at 1700 s^{-1} (Figure 8D and 8E). Moreover, bSM significantly increased platelet secretion on low agonist stimulation, cell scrambling, and thrombus formation at high arterial shear rates.

Discussion

The present study discloses an important role of ASM in the regulation of platelet secretion and initiation of platelet procoagulant activity. According to the present observations, Asm does not appreciably alter agonist-induced increases of platelet cytosolic Ca^{2+} and integrin $\alpha_{IIb}\beta_3$ activation or aggregation, but critically enhances activation-dependent platelet degranulation and platelet-dependent thrombin generation after activation-dependent exposure of platelet phosphatidylserine, resulting in enhanced arterial thrombus formation. Similar to genetic knockout of ASM, pharmacological inhibition of the enzyme with the functional inhibitors amitriptyline or fluoxetine blunted ATP release and thrombus formation. Conversely, ATP release and thrombus formation were augmented in the presence of bSM.

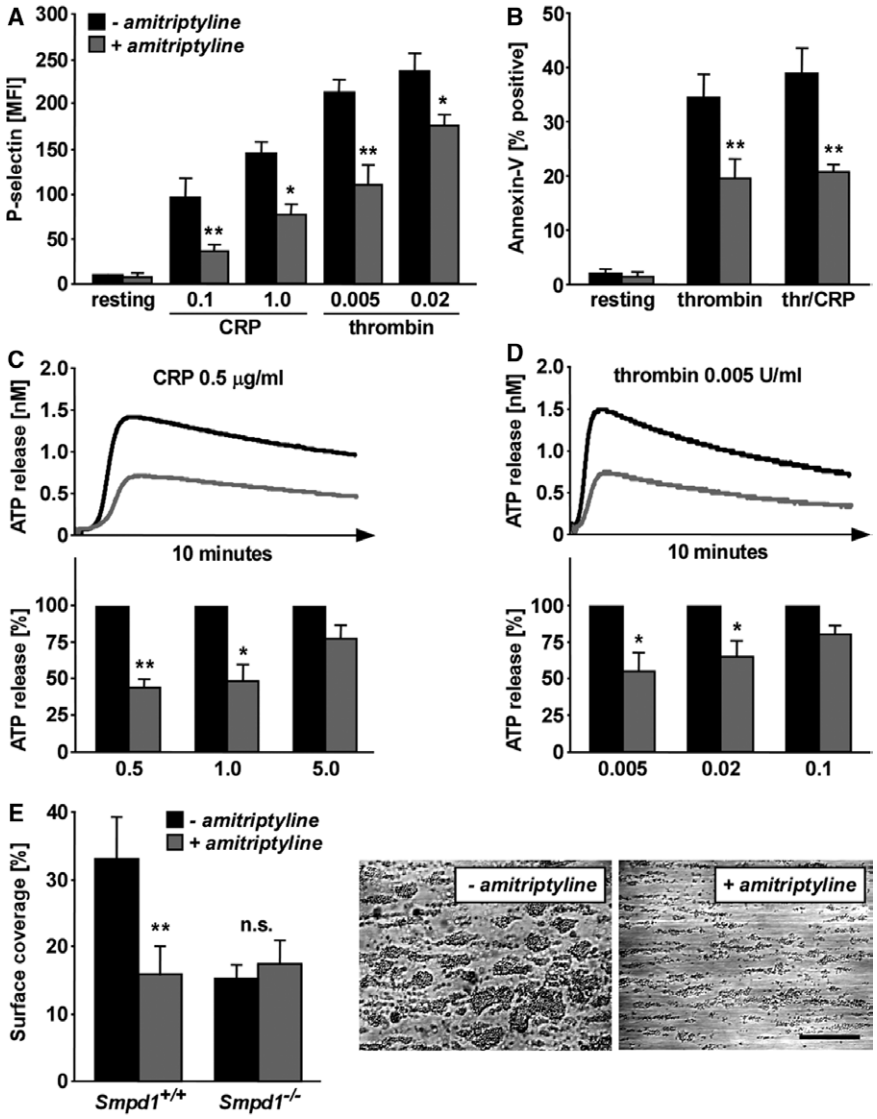

Figure 5. Activation-dependent platelet secretion, phosphatidylserine exposure, and thrombus formation of *Smpd1*$^{+/+}$ platelets in the absence and in the presence of amitriptyline. **A**, Arithmetic means±SEM (n=6) of P-selectin exposure determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ mice in the absence (black bars) and presence (gray bars) of amitriptyline (5 µmol/L) in response to collagen-related peptide (CRP; µg/mL) or thrombin (thr; U/mL) at the indicated concentrations. **B**, Activation-dependent phosphatidylserine exposure of *Smpd1*$^{+/+}$ platelets in the absence and in the presence of 5 µmol/L amitriptyline. Arithmetic means±SEM (n=6) of phosphatidylserine exposure (annexin-V) determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ mice in the absence (black bars) and in the presence (gray bars) of amitriptyline (5 µmol/L) in response to thr (1.0 U/mL) or thr/CRP (0.01 U/mL/5 µg/mL). **C**, Original tracings (**top**) and arithmetic means (**bottom**)±SEM (n=6) of ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with CRP in the absence (black) or presence (gray) of amitriptyline (5 µmol/L). **D**, Original tracings (**top**) and arithmetic means (**bottom**)±SEM (n=6) illustrating the increase of ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with thrombin in the absence (black) or presence (gray) of amitriptyline (5 µmol/L). **E**, Arithmetic means (**left**)±SEM (n=8) and original phase-contrast images (**right**) of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (**left**) and *Smpd1*$^{-/-}$ (**right**) mice in the absence (black bars) and in the presence (gray bars) of amitriptyline (5 µmol/L) over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. Scale bar, 50 µm. **P*<0.05 and ***P*<0.01 indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets treated with the solvent control. n.s. indicates nonsignificant.

The decreased activation-dependent granule secretion and phosphatidylserine exposure in *Smpd1*$^{-/-}$ platelets paralleled their impaired ability to generate adhesion to collagen-coated surfaces under high arterial shear rates in vitro and defective arterial thrombus formation in vivo without affecting stability of formed thrombi. Interestingly, the effect of CRP and thrombin on P-selectin exposure and ATP release was apparent only at low concentrations of the agonists. Increasing the agonist concentration dissipated the differences between *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ platelets, indicating that Asm deficiency decreases the sensitivity of platelets to activating agonists but does not modify platelet function after maximal activation.

Although Asm-deficient platelets showed a significantly diminished activation-dependent secretion, number and morphology of α-granules and dense granules of *Smpd1*$^{-/-}$ platelets were normal and comparable with platelets from wild-type mice, indicating that the observed effects are not because of a regulatory role of Asm in platelet granules biogenesis.

Interestingly, the defects in secretion and phosphatidylserine exposure of *Smpd1*$^{-/-}$ platelets were obviously not secondary to altered increases of cytosolic Ca^{2+} because activation-dependent increase of $[Ca]^{2+}_i$ and store operated Ca^{2+} entry were found to be similar in *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ platelets. This is a remarkable finding because platelet secretion and phosphatidylserine exposure are known to be Ca^{2+}-dependent and, accordingly, suggest that a potential signaling defect is downstream of phospholipase C activation. Furthermore, Ca^{2+}-sensitive integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation were insensitive to sphingomyelinase deficiency.

As shown previously[38] Asm modifies the organization of the plasma membrane and thus participates in the release of cytotoxic granules in T-lymphocytes.[16] Indeed, the cellular process of degranulation and vesiculation is connected with membrane fusion and fission. Asm may have an effect on platelet function by modifying the membrane turnover through the hydrolysis of sphingomyelin.[39] Lack of Asm leads to the reorganization of membrane microdomains.[39] The Asm product ceramide participates in the formation of lipid rafts and cholesterol-enriched membrane microdomains, providing a platform for signaling pathways, such as redox signaling.[40]

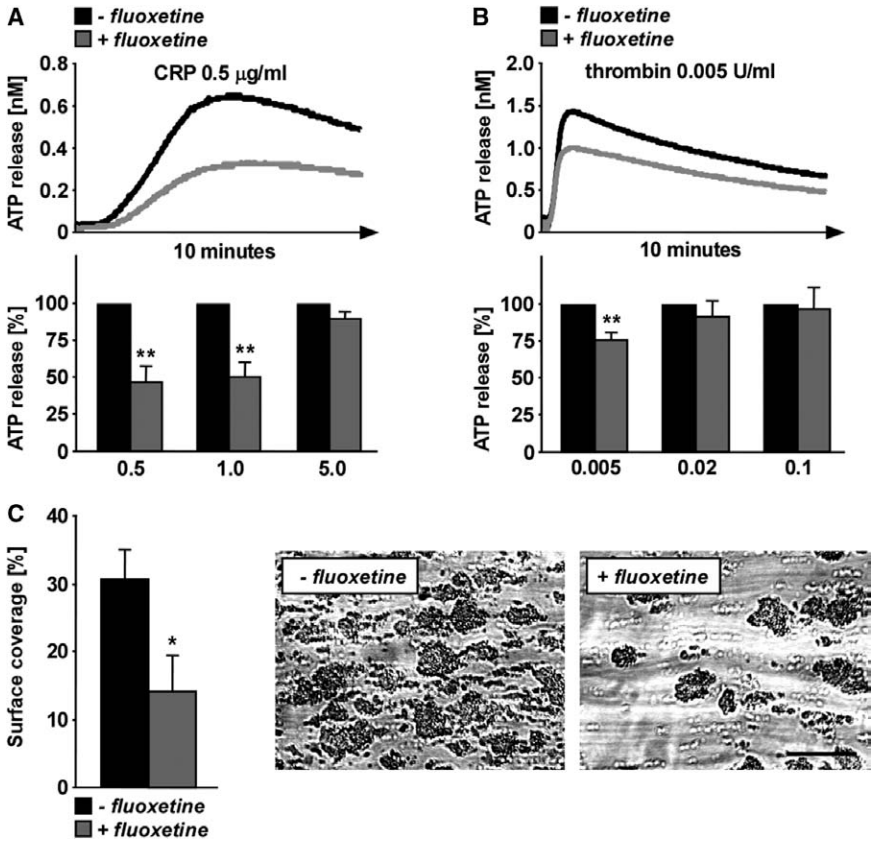

Figure 6. Activation-dependent platelet secretion and thrombus formation of *Smpd1*$^{+/+}$ platelets in the absence and in the presence of fluoxetine. **A**, Original tracings (**top**) and arithmetic means (**bottom**)±SEM (n=6) of ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with collagen-related peptide (CRP) in the absence (black) or in the presence (grey) of fluoxetine (5 μmol/L). **B**, Original tracings (**top**) and arithmetic means (**bottom**)±SEM (n=6) illustrating the increase of ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with thrombin in the absence (black) or in the presence (gray) of amitriptyline (5 μmol/L). **C**, Arithmetic means (**left**)±SEM (n=8) and original phase-contrast images (**right**) of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ mice in the absence (black bars) and in the presence (gray bars) of amitriptyline (5 μmol/L) over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. Scale bar, 50 μm. $^{**}P<0.01$ and $^{*}P<0.05$ indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets treated with the solvent control.

As ceramide increases the curvature and the bending rigidity of the membrane,[18,41] it can change the membrane properties and, therefore, influence the degranulation of cells. Because platelets cannot accomplish de novo synthesis of ceramide,[42] platelets do depend on their sphingomyelinase activity for ceramide production. Sphingomyelinase mediated ceramide generation occurs within seconds,[43] thus influencing rapid activation-dependent cellular functions. As shown in the present study, the ASM seems to play a major role in platelet ceramide metabolism because *Smpd1*$^{-/-}$ platelets showed a significantly decreased ceramide production. Furthermore, we could show that exogenous ceramide was able to rescue the phenotype of defective platelet secretion and thrombus formation.

Asm-dependent regulation of platelet secretion and phosphatidylserine exposure could result from direct regulation of platelet membrane property. As a matter of fact, Asm-deficient platelets showed a significant reduction in activation-dependent thrombin generation because of reduced exposure of phosphatidylserine, the assembly sites for tenase and prothrombinase complexes. Notably, reduced thrombin generation was only found in Asm-deficient platelet-rich plasma, whereas platelet-poor plasma of *Smpd1*$^{-/-}$ mice showed no differences in thrombin levels when compared with that of wild-type samples.

Tricyclic antidepressant medications, including amitriptyline or fluoxetine, are widely used functional experimental inhibitors of Asm because it has been shown that these cationic amphiphiles trigger proteolysis of Asm, resulting in a significantly reduced Asm activity.[38] Treatment with amitriptyline or fluoxetine at concentrations of 5 μmol/L leads to an effective inhibition of Asm activity[44] because granule secretion and phosphatidylserine exposure after stimulation of platelets with CRP or thrombin were significantly impaired in the presence of amitriptyline or fluoxetine, a finding similar to that in Asm-deficient platelets. Treatment of platelets with amitriptyline showed no further inhibitory effect on impaired thrombus formation of *Smpd1*$^{-/-}$ platelets, confirming that there are no significant further targets of amitriptyline affecting platelet function unrelated to Asm inhibition, even though we cannot rule out that amitriptyline and fluoxetine exert additional small effects in platelets.

The addition of bSM could mimic the effect of Asm on platelet degranulation and phosphatidylserine exposure. The stimulation-dependent secretion of platelet α-granules and dense granules shown by P-selectin exposure, ATP release, and phosphatidylserine translocation to the outer leaflet of the plasma membrane was significantly increased in the presence of bSM.

According to the present observations, Asm activity emerged as a critical determinant of platelet function and thrombus formation. Activation of Asm is thus expected to enhance the activation-dependent platelet degranulation and phosphatidylserine translocation by modifying membrane properties. ASM is upregulated or activated by a wide variety of mediators, including thrombin,[37] platelet-activating factor,[30] amyloid,[45,46] and plasminogen activator inhibitor 1.[47] Pathophysiologically, ASM plays a major role in vascular inflammation and the development of atherosclerosis[31] by regulating the release of Weibel–Palade bodies from stimulated endothelial cells.[19] According to the present study, Asm might,

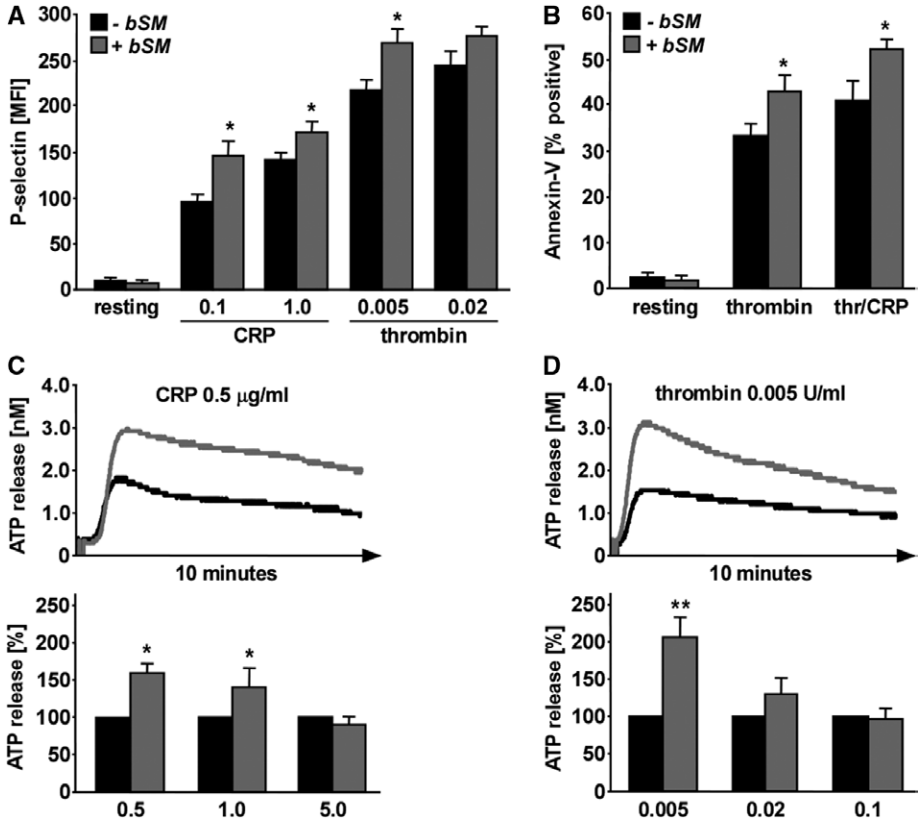

Figure 7. Activation-dependent P-selectin exposure and ATP release from *Smpd1*$^{+/+}$ platelets in the absence and in the presence of bacterial sphingomyelinase (bSM). **A**, Arithmetic means±SEM (n=6) of P-selectin exposure determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ mice in presence (gray bars) or absence (black bars) of bSM (0.01 U/mL) in response to collagen-related peptide (CRP) or thrombin (thr) at the indicated concentrations. **B**, Activation-dependent phosphatidylserine exposure of *Smpd1*$^{+/+}$ platelets in the absence and in the presence of bSM. Arithmetic means±SEM (n=6) of phosphatidylserine exposure (annexin-V binding) determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ mice in the absence (black bars) and in the presence (gray bars) of bSM (0.01 U/mL) in response to thr (1.0 U/mL) and thr/CRP (0.01 U/mL/5μg/mL). **C**, Original tracings (**top**) and arithmetic means (bottom)±SEM (n=6) illustrating the ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with CRP in the absence (black) or in the presence (gray) of bSM (0.01 U/mL). **D**, Original tracings (**top**) and arithmetic means (**bottom**)±SEM (n=6) illustrating the increase of ATP concentration in the supernatant after stimulation of platelets from *Smpd1*$^{+/+}$ mice with thr in the absence (black) or in the presence (gray) of bSM (0.01 U/mL). $^{**}P<0.01$ and $^{*}P<0.05$ indicate statistically significant difference from *Smpd1*$^{+/+}$ platelets treated with the solvent control.

in part, be effective by regulating platelet secretion that plays an active part in vascular inflammation and atherogenesis.[1,4]

After rupture of atherosclerotic lesions with endothelial denudation, circulating platelets are exposed to thrombogenic subendothelial collagen resulting in platelet recruitment to the injured vessel wall.[48] Platelets adhering to collagen, for example, via glycoprotein VI, expose phosphatidylserine at their outer surface and produce phosphatidylserine-exposing membrane blebs and microvesicles propagating the coagulation process by facilitating the assembly and activation of tenase and prothrombinase complex.[49] This conversion of activated platelets to a procoagulant state, referring to the ability of platelets to support thrombin generation, is associated with specific biochemical and morphological changes, some of which are similar to those occurring in apoptotic cells.[50]

Platelets with impaired Asm are less prone to undergo collagen-triggered thrombus formation under high shear stress, a property reversed by adding ceramide or (bacterial) sphingomyelinase. Along those lines, treatment with the functional Asm inhibitors, amitriptyline and fluoxetine, significantly reduced shear stress–induced thrombus formation on collagen.

Platelet degranulation and phosphatidylserine exposure, as described in this article to be modified by Asm, are expected to be the central mechanism underlying thrombus formation under high arterial shear stress.[11,51] In line with these findings, the present study unravels that Asm-dependent regulation of platelet membrane properties, secretion, and platelet-driven thrombin generation plays a significant role for arterial thrombus formation in vivo. Asm-deficient mice were found to be protected against arterial thrombotic occlusion in a model of arterial thrombus formation in mesenteric arterioles after Fe_3Cl-induced injury where thrombus formation is highly thrombin dependent.[52] Accordingly, enhanced activity of ASM could increase platelet responsiveness and platelet-dependent thrombin generation, thus predisposing to thrombotic complications, such as ischemic stroke. Conversely, genetic or pharmacological knockout of ASM may be a therapeutic option to decrease the susceptibility to thrombotic complications. As a matter of fact, Asm-deficient mice displayed a significantly reduced infarct size and a better behavioral outcome after cerebral ischemia.[32]

Despite the strong effect of Asm deficiency on degranulation, as well as thrombin generation and thrombus formation, we could, surprisingly, not observe a significant effect of Asm deficiency on platelet integrin $\alpha_{IIb}\beta_3$ activation and aggregation. In line with these findings, even though thrombus buildup is significantly impaired, thrombus stability is unaffected in *Smpd1*$^{-/-}$ mice. The possibility remains that Asm and Asm-dependent ceramide production may affect platelet membrane structure directly and are involved in the signaling cascade triggering platelet secretion and phosphatidylserine exposure but are not required for platelet integrin $\alpha_{IIb}\beta_3$ activation and aggregation. Further studies will be required to define whether the respective signaling pathways dissociate.

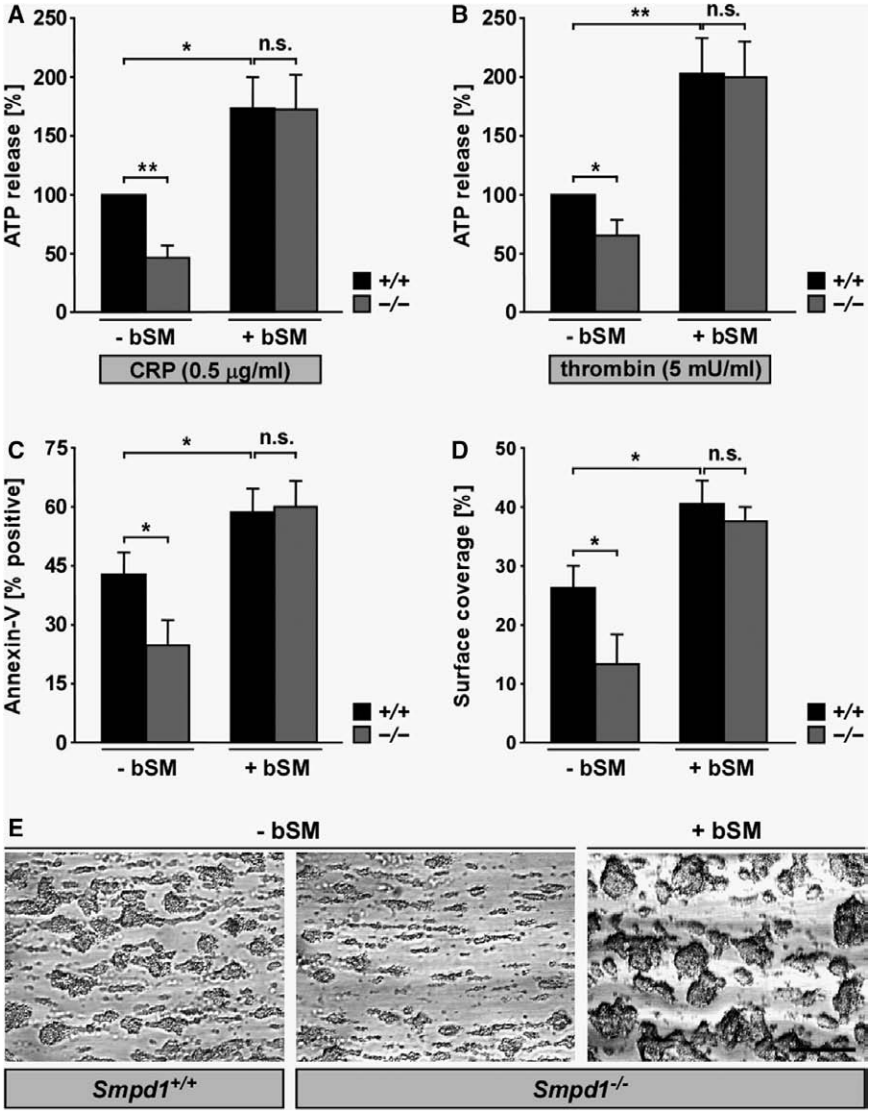

Figure 8. Rescue of defective secretion, cell membrane scrambling, and thrombus formation in *Smpd1*$^{-/-}$ and *Smpd1*$^{+/+}$ platelets by exogenous sphingomyelinase. **A**, Arithmetic means±SEM (n=6) illustrating the ATP concentration in the supernatant after stimulation with 0.5 μg/mL collagen-related peptide (CRP) of platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or presence (**right**) of bacterial sphingomyelinase (bSM; 0.01 U/mL). **B**, Arithmetic means±SEM (n=6) illustrating the increase of ATP concentration in the supernatant after stimulation with 0.005 U/mL thrombin of platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or in the presence (**right**) of ceramide C16 (1 μmol/L). **C**, Arithmetic means±SEM (n=6) of phosphatidylserine exposure (annexin-V binding) determined by flow cytometry in platelets from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (black bars) or in the presence (gray bars) of bSM (0.01 U/mL) in response to thrombin/CRP (0.01 U/mL/5 μg/mL). **D**, Arithmetic means±SEM (n=5) of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ (black bars) and *Smpd1*$^{-/-}$ (gray bars) mice in the absence (**left**) or in the presence (**right**) of bSM (0.01 U/mL) over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. **E**, Original representative phase-contrast images of surface coverage by adherent platelets after perfusion of whole blood from *Smpd1*$^{+/+}$ and *Smpd1*$^{-/-}$ mice in the absence or in the presence of bSM (0.01 U/mL) over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1}. Scale bar, 50 μm. $^{**}P<0.01$ and $^{*}P<0.05$ indicate statistically significant difference.

In conclusion, the present observations identify ASM as a novel regulator of platelet degranulation and thrombus formation. Thus, inhibition of ASM may prove useful in the treatment of thrombosis.

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Disclosures

None.

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Significance

Platelet activation is essential for primary hemostasis and acute arterial thrombosis. In other cell types, both degranulation and phosphatidylserine exposure are modified by sphingomyelinase-dependent formation of ceramide. Platelet secretion and membrane scrambling with phosphatidylserine exposure are crucial to development of arterial thrombosis. The present study thus explored whether acid sphingomyelinase participates in ceramide-dependent regulation of platelet membrane properties and procoagulatory activity. According to the present observations, acid sphingomyelinase plays a decisive role in activation-dependent platelet secretion, phosphatidylserine exposure, and thrombin generation. Acid sphingomyelinase thus participates in signaling mechanisms of platelet adhesion and arterial thrombus formation in vivo. Conversely, treatment with functional acid sphingomyelinase-inhibitors, amitriptyline or fluoxetine, blunted activation-dependent platelet degranulation, phosphatidylserine exposure, and thrombus formation. This study identifies a complete novel signaling pathway regulating platelet membrane properties and function, which has potential therapeutical effect on prevention or treatment of acute thrombotic vascular occlusions underlying myocardial infarction or ischemic stroke.